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Structure and Dynamics of the Phospholamban-SERCA Complex Probed by Site-Directed EPR Spectroscopy

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We are studying the structural dynamics of phospholamban (PLB), a regulator of the SR calcium ATPase (SERCA), by combining site-directed spin-labeling and EPR spectroscopy with solid-phase peptide synthesis. PLB is a 52-residue integral membrane protein that binds and inhibits SERCA in the presence of sub-micromolar calcium concentrations. SERCA activity can be restored without dissociating the two proteins by phosphorylating PLB at Ser16 (Mueller et al., 2004). To observe the effects of phosphorylation and SERCA-binding on PLB conformation, we have synthesized PLB analogs containing the spin-labeled amino acid TOAC. Doubly-labeled mutants were studied by DEER spectroscopy, a pulsed EPR technique capable of measuring interspin distances from 2-7nm. Our results agree with published EPR dynamics data (Karim et al., 2006) showing that PLB equilibrates between an ordered, compact (T) state and an extended, dynamically disordered (R) state. Alone, PLB largely occupies the (T) state, while this equilibrium shifts moderately in favor of the (R) state upon SERCA binding or Ser16 phosphorylation. In contrast, SERCA-bound PLB becomes more ordered and compact upon phosphorylation. We have also used relaxation enhancement EPR with singly-labeled mutants to study the movement of PLB's transmembrane (TM) helix relative to the membrane plane. In these experiments, the spin-lattice relaxation rate of excited spins is enhanced by their collision with paramagnetic relaxation agents (PRAs). For TOAC residues incorporated into the TM domain, PLB motions that reposition this helix will reduce or increase the spin-label's accessibility to water-soluble PRAs, while having the reverse effect for lipid-soluble PRAs. With these experiments, we are constructing a more complete model of PLB dynamics during its interaction with SERCA.

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FRET Detected Interactions of Cardiac Membrane Proteins in Living Cells

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We have investigated the structure of phospholamban (PLB) and its regulation of the sarcoplasmic reticulum Ca-ATPase (SERCA) using fluorescence resonance energy transfer (FRET) on fluorescent fusion proteins expressed in living cells. Fusion proteins were created with either a donor fluorophore (cyan fluorescent protein, CFP), or an acceptor fluorophore (yellow fluorescent protein, YFP), attached to one terminus of the protein of interest. Both N-terminal and C-terminal fusions of CFP and YFP were made to SERCA and N-terminal fusions were made to PLB. These proteins were expressed and co-expressed in either SF21 insect cells or HEK-293 cells, preserving normal physiological function of both proteins. In fluorescence transfer recovery (FTR) experiments, FRET was calculated from the recovery of CFP fluorescence due to photobleaching of YFP. The dependence of donor fluorescence on acceptor photobleaching showed that PLB exists primarily as oligomers in cells but binds to SERCA exclusively as a monomer. Phosphorylation of PLB by PKA, after stimulation of adenylyl cyclase with forskolin, showed a change in FRET. Time-resolved fluorescence showed that the change in FRET was due primarily to a change in the structure of the SERCA-PLB complex, not to a change in protein association. The structural and functional effects of PLB mutation are also under investigation. This work was supported by NIH (GM27906, AR007612).

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Phosphomimetic Mutations Increase FXYD1 Oligomerization, but Does Not Alter its Quaternary Conformation

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Phospholemman (FXYD1, or PLM) is a key regulator of Na^+-K^+ ATPase in the heart, and is principally phosphorylated by cAMP-dependent protein kinase A (PKA) and protein kinase C (PKC). To investigate whether phosphorylation alters FXYD1 structure and oligomerization, we fused cyan or yellow fluorescent protein (CFP/YFP) to the c-terminus of FXYD1 and co-expressed the fusion proteins in AAV-293 cells. Phosphorylation of FXYD1 was mimicked by mutations S68E (PKA site) or S63E/S68E (PKC+PKA sites), and FRET from CFP-FXYD1 to YFP-FXYD1 was quantified by acceptor photobleaching. FRET increased with protein concentration up to a maximum ($FRET_{max}$), which was taken to represent the intrinsic FRET of the bound complex. We did not detect significant changes of $FRET_{max}$ with phosphomimetic mutations, suggesting the quaternary structure of FXYD1 oligomer is not grossly altered by phosphorylation. The concentration dependence of FRET also yielded the relative dissociation constant of the FXYD1 oligomer (K_d), in arbitrary units (AU). Compared to non-phosphorylatable mutant S68A, S68E showed a significant decrease in K_d (14.1 $\pm$ 2.0 and 7.3 $\pm$ 2.0 AU, respectively). The data are consistent with more avid oligomerization of pseudo-phosphorylated FXYD1. Phosphomimetic mutation of both PKC and PKA sites (S63E/S68E) resulted in a K_d of 6.6 $\pm$ 1.1 AU, suggesting there was not a significant additional increase in oligomerization *vs.* the single site mutation. Taken together, the data suggest that phosphorylation can enhance FXYD1 oligomerization without altering the architecture of the oligomeric complex. Increased FXYD1 oligomerization may have an indirect effect on the regulatory interaction of FXYD1 with Na^+-K^+ ATPase.

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Phospholemman Recruits Peroxiredoxin 6 to the Cardiac Sodium Pump

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Phospholemman (FXYD1, PLM), the principal sarcolemmal substrate for protein kinases A and C in the heart, is a regulator of the cardiac sodium pump. We investigated proteins that interact with PLM in adult rat ventricular myocytes using bifunctional crosslinking reagents and co-immunoprecipitation.

Digitonin-permeabilized ventricular myocytes were treated with the heterobifunctional crosslinking reagent sulfo-lc-smpt (distance between reactive groups 2nm, reactive towards amino and sulfhydryl amino acid side chains), and cell lysates immunoblotted for PLM. We found sulfo-lc-smpt quantitatively crosslinked PLM to a 20-25kDa protein (electrophoretic mobility of PLM 15kDa, electrophoretic mobility of crosslinked adduct 37kDa). Co-immunoprecipitation experiments indicated that the crosslinked adduct was PLM linked to the anti-oxidant protein peroxiredoxin 6 (prdx6). PLM phosphorylation at serine 68 (protein kinase A activation with 10μM forskolin) or at serines 63, 68 and threonine 69 (protein kinase C activation with 300nM PMA) had no effect on the ability of sulfo-lc-smpt to crosslink PLM to prdx6. Hydrogen peroxide treatment of ventricular myocytes (1-100μM) was also without effect on the binding of prdx6 to PLM.

In conclusion, our data suggest a new role for PLM in the heart. As well as being responsible for kinase-mediated regulation of the cardiac sodium pump, it is responsible for recruitment of prdx6 to this ion transporter. Prdx6 catalyzes the reduction of hydrogen peroxide, fatty acid hydroperoxides and phospholipid hydroperoxides using glutathione. Given the well-established sensitivity of the alpha subunit of the sodium pump to cysteine oxidation, recruitment of prdx6 to the sodium pump by PLM may be important to maintain pump activity during periods of oxidative stress.

Photosynthesis & Photoreceptors

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Identifying the Quencher of Excited State Energy in Photosynthetic Antennae

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Excess energy dissipation in plants in high-light conditions requires the formation of a quenching site. Although several different quenching mechanisms have been proposed, all of them involve pigment-pigment interactions between chromophores coordinated to the antenna complexes of Photosystem II. The best quencher-candidates are Chlorophyll-Chlorophyll and Chlorophyll-carotenoid pairs, likely belonging to the same Lhcb complex, which switches between a light-harvesting and a dissipative state, in this way changing the strength of the interaction. In principle all the antenna complexes can contain a quencher, as suggested by the analysis of *Arabidopsis* Lhcb-depleted lines, which have shown that none of the Lhcb is *per se* necessary for NPQ, although the absence of all of them leaves the system unprotected. This suggests that more than one antenna complex can act as a quencher and thus should contain a quenching site. Previous proposals have suggested a role for Chl 612 interacting with site L1 and Chl 603 interacting with site L2, but also other Chls located in the proximity of the carotenoids can be a putative quenching site. The spectroscopic properties of most of the Chls coordinated to several Lhcb complexes, their interactions with neighbouring carotenoids and their effect on the excited state lifetimes of the complexes have been investigated by combining mutation analysis with time-resolved spectroscopy. The experiments have been performed both in solution, where the light-harvesting conformation dominates,